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Impact of biodiesel on biodeterioration of stored Brazilian diesel oil

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ABSTRACT

Alternative fuels are receiving considerable attention, especially biodiesel, which is recognized for its environmental benefits. One advantage is its biodegradability. However, biodegradability may allow the fuel to be more susceptible to microbial contamination, especially during storage. The susceptibility to biodeterioration of biodiesel, diesel, and diesel containing 5, 10, and 20% biodiesel was evaluated using fungi isolated from contaminated oil systems. *Paecilomyces* sp. produced the highest biomass in 20% and 100% biodiesel, while *Aspergillus fumigatus* grew best in pure biodiesel. Yeasts had the highest rates of degradation, especially *Candida silvicola*, with 100% degradation of all esters. *Rhodotorula* sp. showed greatest activity for C18:3 (linolenic acid), at 39.4%, followed by C18:1 (oleic acid) and C16 (palmitic acid), at 21% and 15%, respectively, after 7 days of incubation. The results are relevant for the resolution of the decade-long debate on the increase in diesel biodegradability due to the addition of biodiesel.

1. Introduction

The use of biodiesel has been widely encouraged in many countries, and global projections indicate increasing substitution of fossil fuels by biofuels. In addition to the recognized environmental benefits of biodiesel, such as lower emissions of particulate matter and greenhouse-effect gases, and no release of sulfur and volatile aromatic compounds into the atmosphere, biodiesel also has lower toxicity and is more easily biodegraded in the environment (Marchetti et al., 2007; Mariano et al., 2008; Sharma et al., 2008; Murugesan et al., 2009a,b; Demirbas, 2009). Diesel oil is a complex mixture of normal, branched, and cyclic alkanes and aromatic compounds. Biodiesel is defined as a mixture of mono-alkyl esters of long-chain fatty acids (FAME) derived from the transesterification of animal fats and vegetable oils, and its physical and chemical characteristics allow it to be added to diesel (Demirbas, 2009). The chain length and degree of unsaturation can vary in animal fats and vegetable oils for many reasons (DeMello et al., 2007). Soybean oil is the primary feedstock used in Brazil for the production of biodiesel. In EU countries, especially Germany, the mixture reaches 5%, and sometimes biodiesel may even be used in its pure form (Schleicher et al., 2008). In Brazil, its application as an alternative fuel became mandatory from January 2008, when 2% biodiesel was added to diesel according to regulations of the National Petroleum Agency (ANP) (Junior et al., 2009). In 2009 the percentage was raised to 4% and in 2010 to 5%. During storage, chemical and physical changes in the properties of biodiesel and binary mixtures may occur as a result of degradation processes, leading to increase in acidity, potential corrosion, and formation of sediment (Passman and Dobranick, 2005). Despite its many benefits, biodiesel has some vulnerabilities: Due to its chemical structure, it is more susceptible to oxidative, thermal and hydrolytic degradation (Dunn, 2005; Leung et al., 2006; DeMello et al., 2007; Knothe, 2007; Mariano et al., 2008; Junior et al., 2009).

The oxidation stability of various biodiesel esters from different feedstocks may be caused by multiple factors: (1) Molecular structure of the fatty esters: Biodiesel is a blend of fatty acid esters having different molecular structures with varying chain lengths, levels of unsaturation, and conformation. (2) Presence of antioxidants: Antioxidants can be naturally present in the feedstock or can be added during or after processing. These compounds usually prevent radical formation in oils by oxygen or light and subsequent degradation. (3) Presence of impurities and degradation products: Some

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impurities can catalyze the formation of radicals in oils, while other impurities can catalyze the degradation pathways of oils once radicals are formed (Waynick, 2005).

In addition, some of its features increase the biodegradability of the fuel, such as the absence of hydrocarbons, the presence of esters of fatty acids, and its greater affinity for water. According to Passman (2003), its biodegradability is a bonus from the perspective of environmental protection, but it is a problem when it occurs in the fuel systems. An increased biodegradability of diesel/biodiesel blends has been demonstrated in many investigations (Bento et al., 2006; Pasqualino et al., 2006; Mariano et al., 2008; Junior et al., 2009). On the other hand, in some studies (DeMello et al., 2007; Mariano et al., 2008; Owsianiak et al., 2009) the biodegradation of hydrocarbons was not accelerated by the presence of biodiesel. It has been postulated that this observation might be related to the catabolic abilities of the chosen microorganisms toward petroleum hydrocarbons (Junior et al., 2009). The microbial contamination of stored fuels, mainly diesel oil, is a major problem in refineries and distribution systems (Bento and Gaylarde, 1996, 2001; Bento et al., 2004). Many factors, such as the presence of water in the bottom of the tanks during storage, have been cited as increasing microbial growth in the systems and can lead to blocking of pipelines and filters, affecting the final quality of the fuel and corrosion of the tanks (Bento and Gaylarde, 2001; Bento et al., 2004). Good housekeeping, monitoring, and proper usage of an effective biocide are crucial measures for an anti-microbial strategy (Gaylarde et al., 1999; Passman, 2003; Passman and Dobranick, 2005; Siegert, 2009). A concentration of only 1% water in a storage system is sufficient for the growth of microorganisms, bacteria, and yeasts, as well as for the development of fungal biomass at the oil/water interface (Gaylarde et al., 1999; Chesneau, 2000; Bento et al., 2004). Examples of moulds and yeasts isolated from fuels include members of the genera *Candida*, *Rhodotorula*, *Aspergillus*, *Paecilomyces*, *Fusarium*, *Hormoconis*, *Penicillium*, and *Alternaria* (Bento and Gaylarde, 1996, 2001; Gaylarde et al., 1999). Many authors have reported the biodegradability of biodiesel blends in environments such as soil (Mariano et al., 2008; Junior et al., 2009) and water (Zhang et al., 1998; DeMello et al., 2007). Zhang et al. (1998) found that the rate of diesel biodegradation in water can be three times greater in the presence of biodiesel. According to Mariano et al. (2008), although biodiesel is more easily and rapidly biodegraded than diesel oil, among the diesel/biodiesel blends evaluated (2%, 5%, and 20%), only those with the highest proportions of biodiesel were significantly degraded more efficiently than pure diesel in soil. Junior et al. (2009) stated that it has been debated for a decade whether the addition of biodiesel facilitates diesel biodegradability. In addition, the influence of varying the proportions of biodiesel in biodiesel/diesel blends on fungal biodeterioration in tanks has not been investigated. The aim of this work was to assess in the laboratory the effect of adding various proportion of soy-derived biodiesel to diesel oil, on the growth of deteriogenic fungi (moulds and yeasts). The production of biosurfactants and the degradation of esters assessed by chromatography were also investigated.

2. Materials and methods

2.1. Microorganisms

The fungi *Aspergillus fumigatus* and *Candida silvicola* were isolated from the interfacial biomass of diesel storage systems in Brazil (Bento and Gaylarde, 2001); *Paecilomyces* sp. and *Rhodotorula* sp. were isolated from the sediments of biodiesel sampled in a Brazilian bus company. The sediment was plated on malt agar and incubated at 28 °C. After purification by repeated subculture, moulds were identified by macroscopic examination of colonies on malt agar slant tubes and microscopic examination (slide culture technique). Yeasts were identified according to carbohydrate assimilation patterns, using the API 20C 190 AUX (bioMérieux) galleries, which were used according to the manufacturer's recommendations.

2.2. Fuels

The fuels used were diesel oil (0.2% sulfur, metropolitan grade) and biodiesel (from soybean oil), both supplied by the Brazilian Petroleum Company, Ipiranga. Soybean oil is the primary feedstock used in Brazil for biodiesel production. Biodiesel blends were prepared in the laboratory at the following volume percentage compositions of biodiesel/diesel: 0/100, 5/95, 10/90, 20/80, and100/0 – respectively denominated B0, B5, B10, B20, and B100. The fuels and the blends were sterilized by vacuum filtration, using membranes of pore size 0.22 μm, and placed in previously sterilized glass bottles, which were covered with aluminum foil to prevent photo-oxidation of the fuel.

2.3. Growth assays

The mineral medium used (Richard and Vogel, 1999) contained (g L^{-1}): 0.7 KCl, 2.0 KH_2PO_4, 3.0 Na_2HPO_4, 1.0 NH_4NO_3, 4.0 $MgSO_4$, 0.2 $FeSO_4$, 0.2 $MnCl_2$, and 0.2 $CaCl_2$. The sole carbon source was diesel, biodiesel, or the blends described in Section 2.2.

2.3.1. Moulds

Moulds inocula were grown on malt agar at 28 °C for 7 days, and yeasts on GYMP broth (glucose, 20 g l^{-1}; malt extract, 20 g l^{-1}; yeast extract, 5 g l^{-1}; monobasic sodium phosphate, 2 g l^{-1}) for 48 h at the same temperature. After growth, suspensions of mould spores and yeast cells were prepared in distilled water, and counted in a Neubauer chamber. The final concentration used was 10^7 spores ml^{-1} for the moulds, and 10^4 cells ml^{-1} for the yeasts. Mould growth tests were carried out in 150-mL flasks containing 25 ml minimal medium and 25 ml diesel oil (B0), biodiesel (B100), or the blends (B5, B10, B20) as the only carbon source. After 7, 14, 21, 28, 35, 42, and 60 days the biomass of moulds formed at the oil–water interface was filtered (total volume). Ten milliliters of hexane was utilized to remove the residual oil, the filter paper with biomass was dried to a constant weight, and the final weight was recorded.

2.3.2. Ability of yeast to grow in fuel phase (preliminary tests)

Yeasts' ability to grow in diesel oil and blends was evaluated according to Hanson et al. (1993). This method consists of incorporating into the medium an electron acceptor, 2,6-dicholorophenol–indophenol (DCPIP), thus testing the ability of the microorganisms to utilize the hydrocarbon substrate, which is observed when the color of DCPIP changes from blue (oxidized) to colorless (reduced). Each microtitre-plate well was filled with 250 μl of mineral medium (Richard and Vogel, 1999), 10 μl of fuel (diesel oil, B0; pure biodiesel, B100; diesel blends: B5, B10, and B20) and 25 μl of each microbial suspension standardized at 10^4 cells ml^{-1}. All plates were incubated at 28 °C, and fuel concentration was determined according to the change in the color of the culture medium containing DCPIP after 12 h of incubation (Miranda et al., 2007; Junior et al., 2009). Yeast curves were performed in 250-ml flasks containing 160 ml of mineral medium and 1% of biodiesel (B100), or 1% of a mixture of 20% biodiesel in diesel (B20). All replicates were incubated at 28 °C and 120 rpm. The yeasts were serially diluted in distilled water every 24 h (for 186 h, about 7 days) and enumerated as colony-forming units (CFU) on GYMP agar. Five replicates were set up for each mould and three for each yeast and uninoculated controls.

2.4. Aqueous phase analyses

The pH, surface tension, and emulsifying index measured after 24 h (IE 24%) (Cooper and Goldenberg, 1987) were determined after 7, 14, 21, 28, 35, 42, and 60 days of incubation with and without microorganisms. Measurements of surface tension were evaluated using a Gibertini tensiometer. All measurements were made in triplicate with cell-free liquids obtained by filtration through a 0.22 μm Millipore membrane. The aqueous phase was centrifuged at 10,000 rpm for 10 min to perform the triplicate evaluations in medium free of cells and mycelium.

2.5. Biodiesel analyses

After 60 days of incubation, the oil layer from the mineral medium/fuel flasks was extracted using a separating funnel. Esters were analyzed using an HP 5890 II gas chromatograph with an SGE BPX5 bonded phase capillary column (25 m × 0.2 mm, 0.25 mm film thickness), ScanMode 35–450 AMU (atomic mass units), carrier gas helium and interface temperature 250 °C. Split injection was at 20:1, with injection size 1.0 μl. The Mass Selective Detector (HP 5972) temperature was 280 °C and the oven program was: initially at 60 °C, held for 3 min, then ramped at 7 °C min^{-1} to 280 °C and held for 10 min. Control peak-areas were used as the 100% point of reference and test peak-areas were reported as a percentage of control peak-areas. The content of esters and linolenic acid methyl-esters present in the samples of biodiesel was determined using the conditions specified by European EN Standard Method 14103, regulated by Resolution ANP N° 42 (24.11.2004 – DOU 9.12.2004 – 19.4.2005).

2.6. Statistical analyses

Experiments were carried out using three independent replicates. Data were subjected to analysis of variance and the averages were compared by the Tukey multiple range test using $p = 0.05$. The yeast counts were compared by a *t*-test analysis employing the logarithms of the counts. All analyses were performed with the statistical software Statistic version 7.1.

3. Results and discussion

3.1. Growth in laboratory systems

The microorganisms evaluated in this study were able to grow using diesel and biodiesel blends, as the sole source of carbon and energy.

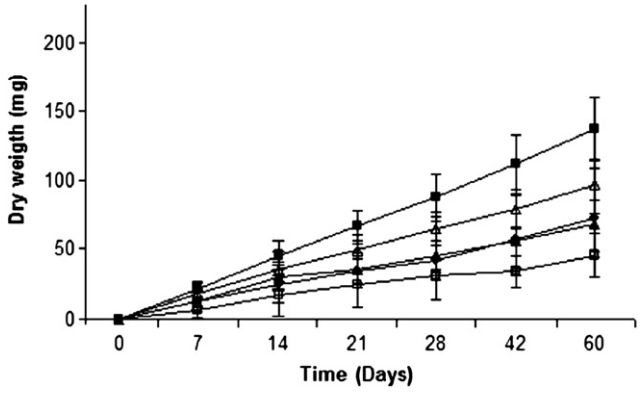

Fig. 1. Growth curve of *A. fumigatus* in mineral medium containing diesel, biodiesel, or different diesel/biodiesel blends. □-B0; ▲-B5; ◆-B10; △-B20; ■-B100.

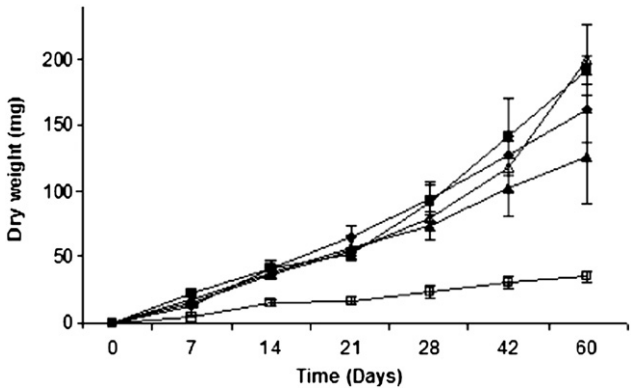

Fig. 2. Growth curve of *Paecilomyces sp.* in mineral medium containing diesel, biodiesel, or different diesel/biodiesel blends. □-B0; ▲-B5; ◆-B10; △-B20; ■-B100.

3.1.1. Moulds

A. fumigatus and *Paecilomyces* sp. produced the greatest biomass after 60 days of growth in pure biodiesel (B100) (Figs. 1 and 2). A lag phase was observed only in pure diesel oil (B0). This initial phase of adaptation is related to the change in growth conditions, as the inoculum was prepared on malt agar, a rich medium. In general, for both moulds the biomass was highest where more biodiesel was present in the blend. However, only B100 differed statistically ($p = 0.05$) from B0 for each sampling time up to 60 days. At the end of 60 days, the *A. fumigatus* biomass was 2.8 times greater in B100 than in B0; the highest biomass values were 140 mg in B100, followed by 100 mg in B20, 74 mg in B10, and 70 mg in B5. At this time, the biomass in B100 differed statistically from B0, B5, and B10 for *A. fumigatus* (Table 1). Thus, biodiesel had positively influenced its growth (Fig. 1). In our experiment the lowest biomass recorded was 48 mg in pure diesel (B0) at 60 days. Bento et al. (2004), using a different mineral medium (Bushnell–Haas) and 10% metropolitan grade diesel (0.5% of sulfur), but with otherwise similar incubation conditions, showed that the same *A. fumigatus* isolate produced 60 mg biomass after 60 days. Although the strain used in this work was isolated from a diesel storage tank and its ability to use this oil as a source of carbon and energy was established, its maintenance on malt agar for 17 years may have led to a reduction in its ability to degrade oil. The method chosen for the storage of microorganisms has a direct influence on the expression of their physiological and biochemical properties, and may even promote genetic changes (Abadias et al., 2001). It may be that the ability to grow on diesel could have been optimized if it had been pre-cultured in a medium containing diesel as sole carbon source before beginning the diesel blend studies. The recalcitrance of some diesel hydrocarbon components is probably caused by the lack of the microbial pathways required to utilize them for growth, and these may have been lost after prolonged storage on non-hydrocarbon media. According to Hill and Hill (2008) each storage process has its own

Table 1
Biomass of *A. fumigatus* and *Paecilomyces* sp. after 60 days growth on various diesel/biodiesel blends.

Fuel-blend	Dry weight (mg)	
	A. fumigatus	*Paecilomyces* sp
B0	47.8[c]	34.6[c]
B5	70.2[c]	123.6[b]
B10	73.8[c]	160.2[a b]
B20	99.0[b]	197.4[a]
B100	145.6[a]	189.0[a]

Same lower caps letters are not statistically different and different lower caps letters are statistically different among treatments by the Tukey test ($p < 0.005$).

characteristics in terms of physical and chemical properties, which exert a selective pressure on the microbial flora prevailing in these niches. There are broad differences between some microbial consortia, and these will be dictated by the chemical and physical characteristics of the niche where they occur.

Paecilomyces sp., which was originally isolated from samples of biodiesel, showed the highest growth throughout the whole incubation time with diesel/biodiesel blends. At the end of 60 days, Paecilomyces sp. biomass was 2.8 times greater in B100 than in B0. The highest biomass values were obtained at the end of 60 days for B100 (140 mg), followed by blend B20 (100 mg), B10 (74 mg), and B5 (70 mg). Unlike the observation made for *A. fumigatus*, where the biomass in B100 differed statistically from B0, B5, and B10 after the 60 days, in the case of Paecilomyces sp. the difference was only statistically significant between the biomasses in B100 and B0 (Table 1 and Fig. 2).

Owsianiak et al. (2009) conducted biodegradation experiments for diesel/biodiesel blends in liquid culture with bacteria. The study revealed that there was an almost linear relation between biodiesel content and biomass production. Bento and Gaylarde (2001), evaluating the ability of various fungi to grow in metropolitan diesel and Bushnell–Haas mineral medium, also showed that *Paecilomyces variotti* did not grow significantly on pure diesel. Biodiesel is more easily metabolized than diesel because it is a natural product; it consists of pure fatty acids, which are readily metabolized through the Krebs cycle (Zhang et al., 1998). Diesel oil is a complex mixture of alkanes and aromatic compounds that requires adapted microorganisms able to produce mono- and dioxygenases that catalyze the initial metabolic step (Marchal et al., 2003; Bento et al., 2004; Junior et al., 2009). In the present study, one has to take into account that the blends contained different proportions of FAMES and that their chain length and degree of unsaturation varied. The biodegradability is a function of chain length, degree of substitution (unsaturation), C=C position, and *cis* and *trans* configurations (Maier, 2000; Demirbas, 2009; Hill and Hill, 2009). Moreover, even fuels of the same specification contain different types and ratios of hydrocarbon components and they may contain additives with differing chemistries (Hill and Hill, 2008, 2009). Rauch et al. (2006) reported substantial biodiversity of both bacteria and fungi in USAF jet fuel tanks, and they observed that the microbial community composition of USAF aviation fuel has changed over time since the initial reports in the 1950s. The changes in microbial community may demonstrate microbial adaptation to changes in aviation fuel composition, additives, and biocide use, or may be the result of improvements of isolation and identification techniques.

3.1.2. Yeasts

Based on results of preliminary investigation using DCPIP to determine *C. silvicola* and *Rhodotorula* sp. growth on the various diesel/biodiesel blends (B0, B5, B10, B20, and B100), we have selected B20 and B100 to pursue the experiment. The growth curves for the two organisms are shown in Figs. 3 and 4.

At the first sampling, after 24 h of incubation, the cells of *C. silvicola* were already in log phase, indicating that in both B20 and B100 the lag phase was shorter than 24 h. In B100 (pure biodiesel) the number of viable cells stabilized from 72 to 120 h, suggesting that the culture had reached its stationary phase. However, the sudden increase in viable cells that was recorded at 144 h remains unexplained and, unfortunately, no other sampling was made between 144 and 186 h to confirm the culture was exhibiting a diauxic type of growth. Nevertheless, in a similar experiment, Miranda et al. (2007) monitored the biomass of *Candida ernobii* and *Rhodotorula aurantiaca* for a period of 20 days in Bushnell–Hass mineral medium containing 2–12% of diesel, and they reported that *R. aurantiaca* exhibited

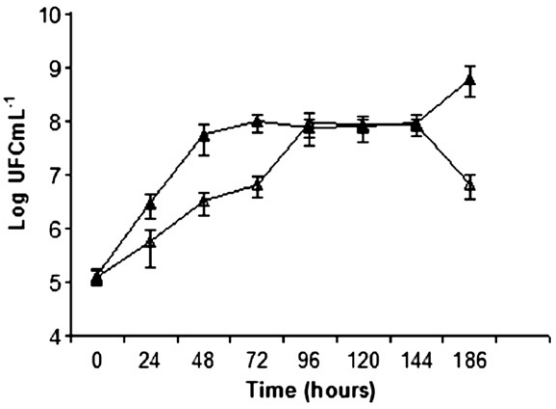

Fig. 3. Growth curve of *C. silvicola* in mineral medium containing biodiesel (B100) or diesel/biodiesel blend B20. △-B20; ▲-B100.

diauxic growth. It is tempting to postulate that this behavior is due to the fact that the diesel oil contains a great diversity of hydrocarbons and the cells might be using different oil components at different phases of growth. However, this hypothesis remains to be demonstrated by molecular data. In the B20 blend, the *C. silvicola* growth curve did not show a diauxic pattern, the stationary phase started at 96 h, and cell viability declined after 144 h.

Rhodotorula sp. and *Candida* sp. have been the most frequently cited (Bento and Gaylarde, 2001; Bento et al., 2004; Miranda et al., 2007; Junior et al., 2009) yeast species able to degrade products derived from oil and diesel oil, kerosene, lubricating oil, and biodiesel. The ability of *C. silvicola* to grow on diesel oil was evaluated by Bento et al. (2004), who reported that yeast growth on diesel reached maximum biomass of 10^8 cells ml^{-1} after 7 days of incubation when the initial concentration was 10^2 CFU ml^{-1}. In our study, the initial concentration of cells for both yeasts was set at 10^5 CFU ml^{-1}. After 7 days, Rhodotorula sp. reached 10^{10} CFU ml^{-1} and *C. silvicola* reached 10^8 CFU ml^{-1} in B100.

3.2. Aqueous phase analyses

3.2.1. Biosurfactant production

The surface tension and emulsifying properties of each culture were determined as described in the Materials and methods section. The IE 24% values for *A. fumigatus* and *Paecilomyces* sp. did not suggest substantial formation of emulsifying agents during growth

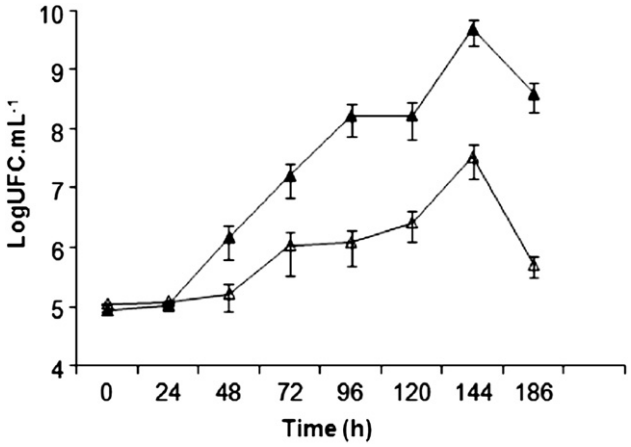

Fig. 4. Growth of *Rhodothorula* in mineral medium containing biodiesel (B100) or diesel/biodiesel blend B20. △-B20; ▲-B100.

on diesel and biodiesel blends (B0, B5, B10, B20, and B100). In addition, there was no substantial reduction of the surface tension when *A. fumigatus* and *Paecilomyces* sp. cultures were compared with their respective uninoculated controls (Table 2). According to DeMello et al. (2007), based on their structural resemblance to surfactants, FAMEs may form ordered associations at the oil–water interface, resulting in a lowering of the oil–water surface tension. This would explain why, for blends B5, B10, and B20, the surface tension recorded after 60 days was slightly lower than the surface tension of the mineral medium alone (53.8 mN m^{-1}).

In the case of *C. silvicola* and *Rhodotorula* sp., a white microemulsion was observed after 7 days of incubation in cultures grown on B100. However, on the basis of the calculated IE 24% values we were unable to conclude that there was production of emulsifying agent by these organisms. After 7 days of incubation, cultures of *C. silvicola* in B100 and B20 showed substantial reduction ($p < 0.05$) in surface tension when compared to uninoculated controls. Similar to the observation reported above with the moulds, the surface tension values for the uninoculated controls for B20 and B100 were lower than the value for the mineral medium alone. In other studies, microorganisms that decrease surface tension from 72 mN m^{-1} (measurement of pure water) to about 35 mN m^{-1} and 40 mN m^{-1} were considered good biosurfactant producers, and those that decrease it to less than 35 mN m^{-1} were considered efficient producers (Bento et al., 2008). In general, biodiesel properties change over time, due to hydrolytic and oxidative reactions. The reduction in surface tension in the aqueous phase might be related to the presence of fatty acid esters (biodiesel), whose chemical structure shares structural features with some surfactants; they usually consist of hydrocarbon chains with one or more fatty acid esters, hydroxyl groups, phosphate, carboxyl, or carbohydrate (Mulligan, 2005).

The production of biosurfactants by species of *Candida* grown on vegetable oil has been reported (Thanomsub et al., 2003; Ilori et al., 2008), and their emulsifying properties have been evaluated. In an aqueous-oil blend, bioemulsifiers are responsible for the formation and stabilization of the emulsion, but they are not necessarily responsible for the lowering of the aqueous phase surface tension (Bento et al., 2008). DeMello et al. (2007) have compared the emulsifying ability of various blends of diesel and biodiesel. They concluded that fatty acid esters increase the stability of the tiny oil drops produced in water, decreasing the water surface tension, and thus reducing the chance of re-aggregation.

Lemos and Pereira (2004) evaluated the production of surfactants by *Aspergillus niger* and *Penicillium corylophilum* in three different media containing soybean oil. After 30 days, the surface tension recorded in each medium differed significantly. For both fungi *P. corylophilum* reduced the surface tension to less than 40 mN m^{-1}, reaching 36.2 mN m^{-1} in the medium containing yeast extract. The fungi investigated in the Lemos and Pereira (2004) study were unable to produce biosurfactants when grown on diesel oil alone or on blends of diesel and biodiesel. The oxidative degradation process, also known as oxidative rancidity, occurs when biodiesel is obtained from oleaginous plants, due to the presence of fatty acid esters (palmitic and linolenic) that are sensible to oxidation (Dunn, 2005; Liang et al., 2006; Cavalcanti et al., 2007; Xin et al., 2009).

3.2.2. Change of pH

The presence of acidic chemicals in a storage tank can affect the final quality of the fuel and lead to internal corrosion of the tanks. In this study, the acidity was evaluated on the basis of the pH value of the aqueous phase. Sixty-day-old cultures of *A. fumigatus* and *Paecilomyces* sp. did not show significant pH variations compared to their respective uninoculated controls, irrespective of the diesel/biodiesel blend used as growth substrate (Table 2).

Santos et al. (2007) isolated *P. variotti* from crude palm oil storage and suggested that the growth of this mould in the oil was the cause of the increased acidity. It has been reported that the low pH in the aqueous phase of contaminated fuels is mainly due to organic acid production and that the rate of acid production is a function of the rate of hydrocarbon degradation (Bento et al., 2004). In our study, the final pH values for both yeasts were similar, at 6.7 in B100 and 6.9 in B20 (Table 2). This suggests for both cases (moulds and yeasts) that there was no substantial acid production, or that there was a buffering effect related to the presence of phosphates (5 g l^{-1}) in the mineral medium. Miranda et al. (2007) evaluated the potential degradation of diesel oil by *C. ernobii* and *R. aurantiaca*. They found no significant pH drop after 7 days in Bushnell–Hass medium for either yeast.

A. fumigatus is a known fuel-deteriogenic mould that reduces the pH of the aqueous phase (Bento and Gaylarde, 2001; Passman, 2003; Bento et al., 2004). In a previous investigation, Bento et al. (2004) have reported that when *A. fumigatus* was grown in Bushnell–Hass mineral medium (2 g l^{-1} phosphate) containing diesel oil as carbon source, the pH of the aqueous phase was reduced from 7.0 to 4.8 after 60 days. Propionic acid was identified, among other metabolites (including alcohols and ketones), in the aqueous phase (Bento et al., 2004). In addition to the metabolites produced during growth on hydrocarbons, the reduction of pH of the aqueous phase may also be caused by cell lysis or by organic acids generated during abiotic degradation of diesel or biodiesel. In the present study the abiotic degradation was assessed from uninoculated controls. Leung et al. (2006) found that the presence of water tends to

Table 2
Surface tension and pH of aqueous phase from cultures of 60 days for moulds and of 7 days for yeasts.

Fungi		Blends									
		B0		B5		B10		B20		B100	
		ST[a]	pH	ST	pH	ST	pH	ST	pH	ST	pH
A. fumigatus	Assay	50 ± 2.3	6.9	45 ± 2.0	6.8	42 ± 0.9	6.8	41 ± 0.4	6.7	40 ± 2.2	6.6
	Control[b]	48 ± 0.3	7.0	45 ± 0.4	6.9	46 ± 0.1	7.0	43 ± 0.0	7.0	42 ± 0.0	6.9
Paecilomyces sp.	Assay	44 ± 1.8	7.2	45 ± 1.4	6.7	46 ± 1.3	6.7	42 ± 0.8	6.5	43 ± 3.6	6.5
	Control	49 ± 0.2	7.2	46 ± 0.6	7.2	43 ± 0.7	7.1	42 ± 1.2	7.1	38 ± 0.7	7.0
C. silvicola	Assay							40 ± 0.1	6.8	35 ± 0.3	6.7
	Control							46 ± 0.0	7.1	43 ± 0.7	7.1
Rhodotorula sp.	Assay							47 ± 0.7	7.0	36 ± 0.1	6.7
	Control							46 ± 0.0	7.1	43 ± 0.7	7.1

[a] ST: Surface tension (mN m^{-1}); Mineral medium measurement: 53.8 mN m^{-1}.
[b] Control: (without microorganisms).

increase the abiotic hydrolytic degradation of biodiesel. Biodiesel may contain various proportions of unsaturated fatty acids, depending on the source material; vegetable oils such as soybean oil, that are rich in oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are unstable when exposed to oxygen, due to the high degree of unsaturation. Moreover, when water is present in the storage systems, hydrolytic decomposition of biodiesel occurs, resulting in the release of free fatty acids, which in turn increases the acidity and subsequently the stability of the biodiesel (Schleicher et al., 2008).

3.3. Oil phase analyses

Degradation of biodiesel (B100) was evaluated from the oil phase of 60-day-old cultures of *A. fumigatus* and *Paecilomyces* sp. and of 168-h-old cultures of *C. silvicola* and *Rhodotorula* sp. The results are shown in Table 3, and they are expressed as the percentage of degradation of each component compared to uninoculated controls. *A. fumigatus* performed better than *Paecilomyces* sp. Both moulds preferentially degraded the C16 (palmitic acid) and C18 (stearic acid) fatty acids; however, these fatty acids were degraded to a greater extent by *A. fumigatus* (around 15% for both esters) than by *Paecilomyces* sp. (0.6% and 3.5%, respectively). *A. fumigatus* is a major contaminant of diesel oil, playing an important role in the formation of biomass at the oil/water interface (Bento et al., 2004). In the present experiments, *A. fumigatus* grew very well in B100, reaching almost three times the biomass produced in B0. The yeast strains exhibited the highest rates of degradation, especially *C. silvicola*, which degraded 100% of all esters. *Rhodotorula* sp. degraded the fatty acids C18:3 (linolenic acid) preferentially, at 39.4%, followed by C18:1 (oleic acid) and C16 (palmitic acid), at 21% and 15%, respectively. Both yeast genera *Candida* and *Rhodotorula* have been cited in the literature as contaminants of diesel oil (Bento et al., 2004; Miranda et al., 2007). Zhang et al. (1998) showed high biodegradability of biodiesel in an aquatic environment, using a mixture of wastewater (sewage) and soil organic matter as inoculum. In this case the degradation exceeded 90% after 28 days. In a similar experiment, Pasqualino et al. (2006) obtained 98% degradation of biodiesel within four weeks when the inoculum was comprised of an activated sludge from a sewage treatment plant. *P. variotti* was found by Santos et al. (2007) to be a major contaminant of crude palm oil during storage. In the current work, our *Paecilomyces* sp. isolate grew well over the 60-day period, but showed a minimal degree of oil degradation (Table 3). A large variety of microorganisms are involved in the biodegradation of biodiesel in various environments, and several investigations provide evidence that they increase the biodegradability of fossil diesel (Zhang et al., 1998; Pasqualino et al., 2006; DeMello et al., 2007; Mariano et al., 2008; Prince et al., 2008; Junior et al., 2009). According to Hill and Hill (2009), fuel-deteriogenic microorganisms grow relatively slowly, and problems will only develop after many months of fuel storage. Biodiesel blends contain enough nutrients, including fatty acid esters, to support microbial growth. Owsianiak et al. (2009), using a consortium of bacteria isolated from an oil-contaminated site, reported complete degradation of methyl-esters in blends of biodiesel fuels, and more than 50% FAME C16 (palmitic acid) was degraded by this consortium. Miller and Mudge (1997) observed that unsaturated (C18) fatty acids were degraded more rapidly than the methyl-esters of saturated fatty acids. DeMello et al. (2007), however, found that esters of saturated C16 fatty acids were degraded faster than the esters of C18. Zhang et al. (1998) stated that in diesel and biodiesel blends in a marine environment, the degradation of esters of fatty acids and n-alkanes occurs simultaneously and faster than other components of diesel; they postulate the existence of a synergy between the degradation of fatty, aliphatic, and aromatic acids. DeMello et al. (2007) and Prince et al. (2008) studied the biodegradation of biodiesel mixtures in a marine environment and in a rainwater retention pond. They showed that the fatty acid methyl-esters were degraded at about the same rate as the n-alkanes and more rapidly than the other diesel components. Several studies concern the biodegradability in aquatic environments and soil of biodiesel and diesel blends. However, we have to consider that some differences are probably related to a combination of different biodiesel stocks used, different microcosm setups (growth conditions), and differences in the challenge populations. These studies also focus on the aspects that make biodiesel an easier and quicker target for degradation by microbial populations found in the environment. This is the first Brazilian study that evaluates growth and degradation of fuel-deteriogenic fungi using diesel, biodiesel, and blends simulating laboratory storage conditions.

Table 3
Percent degradation of biodiesel components by 60 days old cultures of *A. fumigatus* and *Paecilomyces* sp. and by 7 days old cultures of *C. silvicola* and *Rhodotorula* sp.

Biodiesel components	*A. fumigatus*	*Paecilomyces* sp.	*Rhodotorula* sp.	*C. silvicola*
	Degradation (%)			
C16 (palmitic acid)	15.27	0.55	20.99	100
C18 (stearic acid)	15.26	3.54	15.28	100
C18:1 (oleic acid)	7.69	0.47	14.87	100
C18:2 (linoleic acid)	5.46	0.49	11.25	100
C18:3	7.32	0.29	39.64	100

Percentage of degradation was calculated by the following expression: % of degradation = [(Control peak-areas − test peak-areas)/control peak-areas] × 100.

4. Conclusions

There has been considerable debate on the addition of biodiesel and its effects on the biodegradability of diesel. Taken together, our results and those of others discussed here confirm the hypothesis that the addition of FAME to conventional diesel increases the latter's biodegradability. We demonstrated that deteriogenic fungal species were able to degrade five common fatty acids found in soy-derived biodiesel. *C. silvicola* showed the greatest ability to degrade fatty acid esters in biodiesel. Under our assay conditions the moulds and yeasts did not produce surfactants. Similarly, there were no significant reductions in pH caused by any of the tested microorganisms. Microbial growth in stored fuel may lead to economic losses due to blockage of filters, increase of water content, and fuel instability, as well as clogging of fuel nozzles and damage to the engines. Hence it is important to understand the potential hazards of mixing biodiesel with diesel oil.

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